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(54) Title: A PHARMACEUTICAL COMPOSITION COMPRISING A CELL ADHESION MOLECULE

(57) Abstract

A pharmaceutical composition for the prophylaxis or treatment of diseases involving the binding of eosinophils to cells expressing surface vascular cell adhesion molecules comprises a vascular cell adhesion molecule (VCAM) or an anti-VCAM antibody and a pharmaceutically acceptable excipient or carrier. VCAM or anti-VCAM antibodies may also be used to screen for antagonists of VCAM binding to eosinophils.

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A PHARMACEUTICAL COMPOSITION COMPRISING A CELL ADHESION MOLECULE

FIELD OF THE INVENTION

The present invention relates to a pharmaceutical composition comprising a cell adhesion molecule or an antibody capable of binding the molecule, a method of screening for antagonists to the molecule, as well as a method of treatment using said antibody or molecule.

BACKGROUND OF THE INVENTION

It has previously been established that mammalian cells in general express different types of adhesion molecules on their surface mediating cell-to-matrix and cell-to-cell adhesion. Certain adhesion molecules serve specialized functions, one example being adhesion molecules which are expressed on the surface of endothelial cells as a result of cytokine induction, though other forms of induction may also exist. These adhesion molecules interact with structures on the surface of leukocytes so as to mediate the accumulation of white blood cells at sites of inflammation (e.g. caused by infection, injury, autoimmune reactions, allergies or vascular diseases).

The currently known inducible endothelial cell adhesion molecules are composed of an extracellular region which is capable of binding leukocytes, a transmembrane region which is responsible for anchoring the molecule in the cell membrane and which consists of approximately 22-23 amino acids, and a relatively short cytoplasmic region.

Until now, a variety of cell adhesion molecules has been identified. Thus, the intercellular adhesion molecule 1 (ICAM-1) has been cloned and characterized as described in, e.g. D. Simmons et al., Nature 331, 1988, pp. 624-627, EP 289 949, and D.E. Staunton et al., Cell 52, 1988, pp. 925-933, and the

endothelial leukocyte adhesion molecule (ELAM-1) has been identified and cloned as described in, e.g. M.P. Bevilacqua et al., Science 243, 1989, pp. 1160-1165. More recently, a vascular cell adhesion molecule (VCAM-1) has been identified and cloned (L. Osborn et al., Cell 59, 1989, pp. 1203-1211) and its properties described in M.J. Elices et al., Cell 60, 1990, pp. 577-584. This molecule was shown to bind certain lymphocytes and lymphocyte cell lines, but not granulocytes.

SUMMARY OF THE INVENTION

Contrary to what has previously been reported, it has surprisingly been found that the vascular cell adhesion molecule (VCAM) is capable of binding eosinophils. In normal immune response processes, this type of polymorphonuclear granulocyte is triggered to degranulate, i.e. to release its intracellular granules to the outside of the cell. Although this mechanism serves the purpose of combating pathogenic agents which cannot be phagocytosed, recent evidence shows that eosinophils also participate actively in a number of inflammatory diseases such as asthma, ulcerative colitis, rheumatoid arthritis and psoriasis (cf. P. Venge, Agents and Actions 29, 1990, pp. 122-126). Contrary to neutrophil granulocytes, the intracellular secretory granules of eosinophils contain strongly alkaline, cytotoxic substances which are assumed to be responsible for the tissue damage associated with these diseases.

A prerequisite for the accumulation of eosinophils at sites of inflammation is the binding of eosinophils to adhesion molecules, i.e. VCAM, present on endothelial cells. In view of this, it would be of considerable interest for the prohylaxis or treatment of chronic inflammatory conditions to prevent eosinophil binding to VCAMs.

Accordingly, the present invention relates to a pharmaceutical composition for the prophylaxis or treatment of diseases or

conditions involving the binding of eosinophils to cells expressing surface vascular cell adhesion molecules, the composition comprising a vascular cell adhesion molecule (VCAM) and a pharmaceutically acceptable carrier or excipient.

In another aspect, the present invention relates to a pharmaceutical composition for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface VCAMs, the composition comprising an antibody which is capable of binding a VCAM and a pharmaceutically acceptable carrier or excipient.

Diseases or conditions which may be treated by administration of the composition of the invention primarily include allergic diseases or conditions (e.g. asthma, inflammatory bowel disease or dermatitis) and autoimmune diseases (e.g. rheumatoid-arthritis or diabetes).

In the pharmaceutical composition of the invention, the VCAM or the antibody may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for systemic or topical injection or infusion and may, as such, be formulated with a suitable carrier for injection or infusion, such as sterile water or an isotonic saline or glucose solution. The VCAM or antibody may also be formulated with a carrier such as a liposome (this being particularly advantageous for the membrane-bound form of the VCAM variant) or a polypeptide, e.g. albumin or globulin. It is at present assumed that the mode of action of the VCAM on administration is to bind to ligands on the surface of eosinophils, thereby inhibiting eosinophil binding to cells, such as vascular endothelial cells, expressing VCAMs and consequently ultimately preventing the eosinophils from penetrating into extravascular tissue. The antibody, on the other hand, is likely to act by binding to VCAMs expressed on the surface of cells, e.g. vascular

endothelial cells, whereby eosinophil binding to these cells is inhibited.

In a further aspect, the present invention relates to a method of screening for antagonists of VCAM binding to eosinophils, the method comprising incubating a VCAM or a cell capable of expressing a VCAM with a substance suspected to be an antagonist of VCAM binding and subsequently with eosinophils or a ligand derived from eosinophils and capable of binding VCAMs, and detecting any binding of the eosinophils or ligand to the VCAM, decreased binding indicating that said substance is an antagonist of VCAM binding.

In an alternative method of screening for antagonists of VCAM binding to eosinophils, eosinophils or a ligand derived from eosinophils and capable of binding VCAMs may be incubated with a substance suspected to be an antagonist of VCAM binding and subsequently with a VCAM or a cell capable of expressing VCAMs, and any binding of the eosinophils or ligand to the VCAM is detected, decreased binding indicating that said substance is an antagonist of VCAM binding.

DETAILED DESCRIPTION OF THE INVENTION

For pharmaceutical purposes, the VCAM is preferably in soluble form. This may be produced by truncating or substantially deleting DNA sequences of the molecule encoding the transmembrane and cytoplasmic regions of the VCAM. Alternatively, a soluble VCAM may be prepared by introducing a termination codon in the DNA sequence encoding the VCAM at a site upstream of the DNA sequences coding for the transmembrane and cytoplasmic regions. This facilitates the production of the VCAM since it will be secreted from cells containing the truncated DNA sequence substantially only encoding the extracellular region of the VCAM and may readily be isolated and purified from the culture medium of said cells rather than by the more cumbersome process of cell extraction.

Furthermore, a higher purity of the resulting VCAM is more easily achieved when it is in soluble form which facilitates the formulation of pharmaceutical compositions including the molecule. Apart from this, the soluble VCAM variant may suitably be used to screen for VCAM antagonists by procedures involving standard assays (e.g. bound to a chromatographic column or other solid support, as described in further detail below).

It has recently been found (cf. WO 90/13300) that VCAM exists in two forms, one comprising six immunoglobulin (Ig)-like domains (as described by L. Osborn et al., op. cit.), and a variant form comprising seven Ig-like domains. The present inventors currently believe the variant form to be the one most commonly expressed by vascular cells, and therefore most important for the present purpose. In a preferred embodiment, the pharmaceutical composition of the invention therefore includes the VCAM variant comprising seven Ig-like domains, or a derivative thereof.

The term "derivative" is used to indicate a polypeptide which is derived from the native VCAM variant by suitably modifying the DNA sequence coding for the variant, resulting in the addition of one or more amino acids at either or both the C- and N-terminal ends of the native amino acid sequence, substitution of one or more amino acids at one or more sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native sequence or at one or more sites within the native sequence, or insertion of one or more amino acids in the native sequence. It is understood that such derivatives should retain characterizing portion(s) of the native VCAM variant, in particular the additional Ig-like domain or a portion thereof.

The VCAM variant was initially cloned from endothelial cells and identified by sequencing. Like the previously described VCAM-1, the variant molecule has been found to exhibit amino

acid sequence and structural similarity to proteins of the Ig gene superfamily which are characterized by the presence of one or more Ig-like domains, each consisting of a disulfide-bridged loop which has a number of antiparallel β-pleated strands arranged in two sheets. Although molecules belonging to the Ig gene superfamily have a variety of functions, all cell membrane-bound forms are believed to play an important part in mediating cell surface recognition (for a more detailed description of the structure and function of Ig superfamily molecules, see A.F. Williams and A.N. Barclay, Ann. Rev. Immunol. 6, 1988, pp. 381-405, and T. Hunkapiller and L. Hood, Advances in Immunology 44, 1989, pp. 1-63). By comparing the amino acid sequences of VCAM-1 and the VCAM variant, it has been established that the additional Ig-like domain in the variant molecule occurs as the fourth such domain in the sequence, and that domains 1 and 4 as well as domains 2 and 5, and domains 3 and 6 exhibit a high degree of similarity in their respective amino acid sequences.

A preferred VCAM variant for the present purpose is one which includes amino acid sequence shown in Fig. 1 A-E or Fig. 3 A-D appended hereto, or a derivative thereof (as defined above).

It is currently assumed that each Ig-like domain may have a specific function in the VCAM variant molecule and that VCAM variants with different properties may be produced by interchanging and/or deleting one or more of the Ig-like domains. It is therefore contemplated that, in other VCAM variants useful for the present purpose, the amino acid sequence substantially corresponding to the additional Ig-like domain may equally be located in place of any one of the Ig-like domains 1, 2, 3, 5, 6 or 7 of the native sequence. Likewise, the order of the Ig-like domains 1-7 may be changed. Furthermore, one or more of the Ig-like domains 1, 2, 3, 5, 6 and 7 or parts thereof may be deleted, the deletion optionally being the result of alternative splicing. Thus, deletions may occur at either the N- or C-terminal end of the molecule or

within the sequence. Thus, it may be envisaged that the VCAM variant may be one with N-terminal deletions in the Ig-like domain 1 or a part thereof, Ig-like domains 1-2 or a part thereof, or Ig-like domains 1-3 or a part thereof.

The VCAM variant may most conveniently be prepared by introducing a DNA construct which comprises a DNA sequence encoding the VCAM variant into a suitable recombinant expression vector, and transforming a suitable cell with said recombinant expression vector. The transformed cell may then be cultured in a suitable nutrient medium under conditions which are conducive to the expression of the VCAM variant, and the VCAM variant may be recovered from the culture.

The DNA construct is preferably one which comprises the DNA sequence shown in the appended Fig. 1 A-E or Fig. 3 A-D or a suitable modification thereof. Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the VCAM variant, but which correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different protein structure without, however, impairing the properties of the native variant. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence, or deletion of one or more nucleotides at either end or within the sequence.

Another preferred DNA construct is one which encodes a soluble form of the VCAM variant. This may for instance be obtained by truncating or substantially deleting the DNA sequences of the DNA construct coding for the transmembrane and cytoplasmic regions of the VCAM variant. Alternatively, the DNA construct may comprise a termination codon at a site upstream of the DNA sequences coding for the transmembrane and cytoplasmic regions.

The DNA construct encoding the VCAM variant may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA construct may also be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the VCAM variant of the invention by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989). In this case, a genomic or cDNA sequence encoding the VCAM variant may be modified at a site corresponding to the site(s) at which it is desired to introduce amino acid substitutions, e.g. by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures.

Finally, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques. The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

The recombinant expression vector into which the DNA construct is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of

vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the VCAM variant should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the VCAM variant in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding the VCAM variant may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3

(McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for the VCAM variant of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The host cell into which the expression vector is introduced may be any cell which is capable of producing the VCAM variant and is preferably a eukaryotic cell, in particular a mammalian cell. Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells (including yeast cells) may be used as host cells. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of Saccharomyces cerevisiae. Examples of other fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 272 277.

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

If the VCAM variant produced by the cells is one which is deleted of or truncated in the transmembrane and cytoplasmic regions of the native variant, it will be secreted to the growth medium and may be recovered from the medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

If the VCAM variant has retained the transmembrane and (possibly) the cytoplasmic region of the native variant, it will be anchored in the membrane of the host cell and may either be purified therefrom by conventional purification procedures, or the cells carrying the VCAM variant may be used as such in the screening assay. As noted above, however, it may be preferred for some applications that the VCAM variant is in soluble form.

The anti-VCAM antibody is preferably one which is raised against an epitope at least partially included in the following amino acid sequence

Glu Lys Pro Phe Thr Val Glu Ile Ser Pro Gly Pro Arg Ile Ala Ala Gln Ile Gly Asp Ser Val Met Leu Thr Cys Ser Val Met Gly Cys Glu Ser Pro Ser Phe Ser Trp Arg Thr Gln Ile Asp Ser Pro Leu Asn Gly Lys Val Arg Ser Glu Gly Thr Asn Ser Thr Leu Thr Leu Ser Pro Val Ser Phe Glu Asn Glu His Ser Tyr Leu Cys Thr Val Thr Cys Gly His Lys Lys Leu Glu Lys Gly Ile Gln Val Glu Leu Tyr Ser

This sequence substantially corresponds to the additional Ig-like domain of the native VCAM variant as described above, thus ensuring specificity of the antibody against the variant. For this reason, it is preferred that the antibody is a monoclonal antibody or a fragment thereof, such as a $F(ab')_2$ or Fab' fragment, prepared as described in e.g. A. Johnstone and R. Thorpe, Immunochemistry in Practice, 2nd Ed., Blackwell Scientific Publications, 1987, pp. 35-43.

In the screening method of the invention, the VCAM, in particular the VCAM variant, or the anti-VCAM antibody may be immobilized on a solid support. Alternatively, the VCAM or anti-VCAM antibody may be provided with a suitable label. The VCAM may either be used in soluble form immobilized on a solid support, or it may be used in membrane-bound form, i.e. bound to whole cells or as a component of membrane preparations.

The solid support employed in the screening method of the invention preferably comprises a polymer. The support may in itself be composed of the polymer or may be composed of a matrix coated with the polymer. The matrix may be of any suitable material such as glass, paper or plastic. The polymer may be selected from the group consisting of a plastic (e.g. latex, a polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylalcohol, nylon, polyvinylacetate, and any suitable copolymer thereof), cellulose (e.g. various types

of paper, such as nitrocellulose paper and the like), a silicon polymer (e.g. siloxane), a polysaccharide (e.g. agarose or dextran), an ion exchange resin (e.g. conventional anion or cation exchange resins), a polypeptide such as polylysine, or a ceramic material such as glass (e.g. controlled pore glass).

The physical shape of the solid support is not critical, although some shapes may be more convenient than others for the present purpose. Thus, the solid support may be in the shape of a plate, e.g. a thin layer or microtiter plate, or a film, strip, membrane (e.g. a nylon membrane or a cellulose filter) or solid particles (e.g. latex beads or dextran or agarose beads).

The label substance with which the VCAM or anti-VCAM antibody may be labelled is preferably selected from the group consisting of enzymes, coloured or fluorescent substances, radioactive isotopes and complexing agents.

Examples of enzymes useful as label substances are peroxidases (such as horseradish peroxidase), phosphatases (such as acid or alkaline phosphatase), ß-galactosidase, urease, glucose oxidase, carbonic anhydrase, acetylcholinesterase, glucoamylase, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase, ß-glucosidase, proteases, pyruvate decarboxylase, esterases, luciferase, etc.

Enzymes are not in themselves detectable but must be combined with a substrate to catalyse a reaction the end product of which is detectable. Examples of substrates which may be employed in the method according to the invention include hydrogen peroxide/tetramethylbenzidine or chloronaphthole or o-phenylenediamine or 3-(p-hydroxyphenyl) propionic acid or luminol, indoxyl phosphate, p-nitrophenylphosphate, nitrophenyl galactose, 4-methyl umbelliferyl-D-galactopyranoside, or luciferin.

Alternatively, the label substance may comprise coloured or fluorescent substances, including gold particles, coloured or fluorescent latex particles, dye particles, fluorescein, phycoerythrin or phycocyanin.

Radioactive isotopes which may be used for the present purpose may be selected from I-125, I-131, In-111, H-3, P-32, C-14 or S-35. The radioactivity emitted by these isotopes may be measured in a gamma-counter or a scintillation camera in a manner known per se.

Complexing agents which may be employed for the present purpose may be selected from biotin (which complexes with avidin or streptavidin), avidin (which complexes with biotin), Protein A (which complexes with immunoglobulins) and lectins (complexing with carbohydrate receptors). As the complex is not directly detectable, it is necessary to label the substance with which the complexing agent forms a complex. The labelling may be carried out with any one of the label substances mentioned above for the labelling of the enzyme.

The eosinophil-derived ligand capable of binding VCAM, which ligand may be used in the screening method of the invention, may be used in isolated form and may, as such, be provided with a label or may be immobilized on a solid support, respectively, as described above. However, the ligand may also be used in membrane-bound form, i.e. bound to whole cells or as a component of membrane preparations. If the ligand is bound to whole cells (expressed on their surface), binding of the ligand to VCAM may be measured by counting the cells visually, or by measuring naturally occurring intracellular enzyme activity, e.g. peroxidase activity, or by measuring enzyme activity introduced into the cells by recombinant DNA techniques. An example of a useful ligands is very late antigen-4 (VLA-4) (a molecule which acts as a specific ligand for VCAMs on the surface of eosinophils (and other leukocytes)).

The present invention also relates to a method of preventing or treating diseases or conditions involving the binding of eosinophils to cells expressing VCAMs, the method comprising administering, to a patient in need thereof, an effective dosage of a VCAM or anti-VCAM antibody, as described above.

The invention further relates to the use of VCAM, an anti-VCAM antibody or a VCAM antagonist identified by the method of the invention for the preparation of a medicament for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing VCAMs. As indicated above, it is preferred that the VCAM used for this purpose is in soluble form.

It is furthermore contemplated to locate the ligand-binding site on the VCAM variant of the invention, for instance by preparing deletion derivatives of the native VCAM variant (as described above) and incubating these with ligands known to bind the full-length VCAM variant (e.g. an anti-VCAM antibody, VLA-4 or cells expressing VCAM ligands on their surface) and detecting any binding of the ligand to the VCAM deletion derivative. Once the ligand-binding site has been located, this may be used to aquire further information about the three-dimensional structure of the ligand-binding site. Such three-dimensional structures may, for instance, be established by means of protein engineering, computer modelling and/or crystallographic techniques. Based on the three-dimensional structure of the ligand-binding site, it may be possible to design substances which are antagonists of intercellular binding by binding to VCAM and which have a three-dimensional structure substantially complementary to that of the ligand-binding site.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated in the following examples with reference to the appended drawings in which

Fig. 1 A-E shows the cDNA sequence of the native full-length VCAM variant of the invention and the deduced amino acid sequence,

Fig. 2 shows a comparison between the 93 amino acid additional Ig-like domain of the VCAM variant of the invention (top) and the first part of the published VCAM sequence (bottom), and

Fig. 3 A-D shows the cDNA sequence of a soluble VCAM variant of the invention and the deduced amino acid sequence.

The present invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Construction of a cDNA encoding a VCAM variant with 7 Ig-like domains

Human umbilical vein endothelial cell cultures were established essentially by the method described by Jaffe et al., 1973. J. Clin. Invest. 52: 2745-2756. Cultures in low passage numbers were treated with phorbol myristate acetate (PMA) at a concentration of 50 nM for 3-7 hours, after which the cells were harvested and frozen. Total RNA from the cells were prepared by a single-step guanidinium thiocyanate-phenol-chloroform extraction procedure (P. Chomczynski and N. Sacchi. 1987. Anal. Biochem. 162: 156-159). First strand cDNA was synthesized using 7 μg of total RNA per experiment. For some experiments first strand synthesis was primed by a 17-mer oligo dT primer, while for other experiments a specific VCAM 3' oligonucleotide primer 5'-ATCAAGACTAGTCTACACTTTTGATTTCTGTGCTTC-3' was used. Reagents used for synthesis of first strand cDNA by reverse transcription was from a commercial cDNA synthesis kit (Riboclone cDNA synthesis system, Promega Corporation, Madison, WI, USA) and reaction conditions were as recommended by the manufac-

turer.

VCAM cDNA fragments were prepared from first strand cDNA by polymerase chain reaction using specific oligonucleotide primers (R.K. Saiki et al. 1988. Science 239: 487-491). PCR was performed using the Gene Amp kit (Perkin Elmer Cetus, Norwalk, CT, USA).

In each PCR, approximately 1-3 µg of reverse transcribed mRNA was used as template. The following specific primers were used for PCR:

1. 5'-CAG CAA GGT ACC ATG CCT GGG AAG ATG GTC GTG ATC C-3'
2. 5'-AAG GTG CTG CAG ATT CCC ATT ATC TAA TTT CTT ACT-3'
3. 5'-GAA ATT AGA TAA TGG GAA TCT GCA GCA CCT TTC TGG A-3'
4. 5'-ATC AAG ACT AGT CTA CAC TTT TGA TTT CTG TGC TTC-3'

Mismatches were introduced in the primers compared with the published VCAM cDNA sequence (L. Osborn et al. 1989. Cell 59: 1203-1211) thus creating endonuclease restriction sites to facilitate subcloning and assembly into appropriate cloning vectors of amplified cDNA fragments. Two of these sites (primer 1: a KpnI site, primer 4: a SpeI site) were located outside the protein coding region, while the other two (primers 2 and 3: PstI sites) did not affect the amino acid sequence of the protein encoded by the constructed cDNA. Each PCR reaction cycle comprised denaturation of the template at 94°C for 1 minute, annealing of the primers to the templates for 2 minutes at 50°C, followed by extension of the primers for 3 minutes at 72°C. This cycle was repeated 25 times, resulting in specific VCAM cDNA fragments.

When PCR was performed using primers 1 and 2, a fragment of the expected size (~800 bp) resulted. Surprisingly, however, when PCR was performed with primers 3 and 4, a fragment of approximate size 1400 bp (expected: 1162) resulted. The experiment was repeated several times with the same result. PCR performed with

primers 1 and 4 resulted in a fragment of approximately 2200 bp compared to the expected size of 1968 bp.

The isolated cDNA fragments were digested with the endonucleases (New England Biolabs, MA, USA) KpnI and PstI (primer combination: 1 and 2) or PstI and SpeI (primer combination 3 and 4), and the two fragments were subcloned separately into the pBluescript II KS+ vector (Stratagene, CA, USA) by the method described by Sambrook et al. (Molecular cloning. A laboratory manual (J. Sambrook, E.F. Fritsch and T. Manuatis, eds.), Cold Spring Harbor Laboratory Press, 1989). Cells of E. coli strain XL-1 Blue (Stratagene, CA, USA) were made competent according to the method of Hanahan (1983. J. Mol. Biol. 166: 557-580) and used for transformation with the vectors indicated above. The VCAM cDNA was assembled from two subcloned cDNA fragments (KpnI-PstI, PstI-SpeI) according to the method described by Sambrook et al. (Molecular Cloning. A laboratory Manual (J. Sambrook, E.F. Fritsch and T. Maniatis, eds.), Cold Spring Harbor Laboratory Press, 1989) in the mammalian cell expression vector Zem219b (described in DK Patent Application No. 3023/88) in which the oligonucleotide 5'-GATCCGGTACCT-3' had been inserted between the BamHI and the first XbaI site resulting in the introduction of a KpnI cloning site. This VCAM expression vector was termed pVCAM-exp.

The cDNA fragments were sequenced by the enzymatic chain termination method described by Sanger et al., Proc. Natl. Acad. Sci. USA. 1977. 74: 5463-5467, using T7 DNA polymerase (Sequenase Kit, USB, Cleveland, Ohio, USA).

The DNA sequence of VCAM cDNA isolated as described is shown in Fig. 1 A-E. The sequence was identical to the published sequence (L. Osborn et al., 1989. Cell 59: 1203-1211) except for three base differences (A -> G in position 269, T -> A in position 792 due to the introduction of a PstI site, and A -> G in position 1790 resulting in Gln -> Arg) and the unexpected finding of additionally 276 bases after base 928 between C and

G in an alanine codon resulting in the loss of an alanine residue and the addition of 93 amino acid residues to the published sequence. This localization of the additional amino acids corresponds exactly to the transition between immunoglobulin domain 3 and 4 of the amino acid sequence encoded by the published VCAM cDNA sequence.

Using the GAP function of the University of Wisconsin, Genetics Computer Group programme (J. Devereux et al., 1984. Nucleic Acids Res. 12: 387-395), the amino acid sequence encoded by the cDNA insertion was compared to all five immunoglobulin domains encoded by the published VCAM cDNA sequence. Fig. 2 shows that a very high degree of identity (72 %) was found to the first Ig domain of VCAM.

Taken together the sequence data presented definitively identifies a hitherto unknown molecular form of the VCAM molecule with an additional seventh Ig domain.

Example 2

Construction of a cDNA encoding a soluble VCAM variant with 7 Ig-like domains

Using the cloned VCAM cDNA inserted in the mammalian cell expression vector Zem219b (see **Example 1**) as a template a cDNA encoding a soluble form of the VCAM molecule was constructed by polymerase chain reaction (PCR) using specific oligonucleotide primers (R.K. Saiki et al., 1988. Science 239: 487-491). PCR was performed using the Gene Amp kit (Perkin Elmer Cetus, Norwalk, CT, USA) according to the manufacturer's instructions.

The PCR was performed using 50 ng of the pVCAM-exp plasmid as template. The following specific primers were used:

1. 5'-CAG CAA GGT ACC ATG CCT GGG AAG ATG GTC GTG ATC C-3'
2. 5'-CAC GAG ACT AGT CTA AGA AAA ATA GTC TTT GTT GTT TTC-3'

Mismatches were introduced in primer 2 compared with the published VCAM cDNA sequence (L. Osborn et al. 1989. Cell 59: 1203-1211) thus creating an endonuclease restriction site (SpeI) to permit subcloning into the Zem219b (described in DK Patent Application No. 3023/88) expression vector and furthermore creating a translation stop codon resulting in deletion of the putative transmembrane and cytoplasmic domains of the encoded VCAM protein. Each PCR reaction cycle comprised denaturation of the template at 94°C for 1 minute, annealing of the primers to the templates for 2 minutes at 50°C, followed by extension of the primers for 3 minutes at 72°C. this cycle was repeated 10 times.

The isolated approximately 2.2 kb cDNA fragment was digested with the endonucleases (New England Biolabs, MA, USA) KpnI and SpeI and subcloned into the Zem219b mammalian cell expression vector by the method described by Sambrook et al. (Molecular cloning. A laboratory manual (J. Sambrook, E.F. Fritsch and T. Maniatis, eds.), Cold Spring Harbor Laboratory Press, 1989). The sequence of the subcloned cDNA was verified by the enzymatic chain termination sequencing method described by Sanger et al., Proc. Natl. Acad. Sci. USA. 1977. 74: 5463-5467, using T7 DNA polymerase (Sequenase Kit, USB, Cleveland, Ohio, USA) and found to be identical to the sequence described in Example 1 except for the presence of the termination codon introduced into the sequence.

The DNA sequence of the cDNA encoding a putative soluble form of VCAM with 7 Ig domains isolated as described is shown in Fig. 3 A-D together with the deduced amino acid sequence.

Example 3

Adherence assay of HL-60 cells and COS-7 cells transfected with a cDNA encoding wild type VCAM with 7 Ig homology units

Materials and methods

Cell lines: COS-7 cells (ATCC CRL 1651) were cultured in DMEM containing 10% FCS, 100 U/ml of penicillin and 100 µg/ml of streptomycin.

HL-60 cells (ATCC CCL 240) were cultured in RPMI 1640 medium containing 10% FCS, 100 U/ml of penicillin and 100 µg/ml of streptomycin.

DNA constructs: The mammalian expression vector Zem 129b is described in DK Patent Application No. 3023/88.

The wild-type VCAM cDNA encoding seven Ig homology units were inserted into Zem 219b as described in Example 1 resulting in the expression plasmid pVCAM-exp.

Transfections: Transfection of COS cells in Petri dishes was performed with the plasmids Zem 219b and pVCAM-exp using the calcium phosphate technique (F.L. Graham and A.J. van der Eb. 1973. Virology 52: 456-467) with the modifications described in: "DNA Cloning, A Practical Approach" (Glover, D.M.), vol. I + II. 1985. IRL Press. Twenty µg of DNA was used per transfection. Sixteen hours post transfection the media were changed, and 32 hours later the transfected cells were used for adherence assay.

Adherence assay: Transfected COS-7 cells were washed once with RPMI 1640 containing 1% FCS (assay medium). HL-60 cells were washed once with assay medium, and 6 x 10^6 cells in a total volume of 3 ml were applied to each COS-7 Petri dish. After 30 min incubation at room temperature the cells were washed twice with assay medium, and the result evaluated.

Results: Visual evaluation after transfection and adherence assay showed a very low level of HL-60 binding to mock transfected (Zem 219b) COS cells, while the major part of HL-60 cells present adhered to COS cells transfected with pVCAM-exp.

These results demonstrate the ability of VCAM cDNA encoding 7 Ig homology units to encode a protein with adhesive properties towards a leukocyte cell line.

Example 4

Adherence assay of baby hamster kidney cells expressing VCAM comprising 7 Ig-like domains and eosinophil and neutrophil granulocytes

Baby hamster kidney cells (ATCC CCL 10) were transfected with the cloned VCAM cDNA inserted into the mammalian cell expression vector Zem219b (see Example 1) using a modification of the calcium phosphate coprecipitation technique (C. Chen and H. Okayama, Mol. Cell. Biol. 7, 1987, pp. 2745-2752). 0.25 x 10^6 cells were seeded in DMEM containing 10 % FCS, 100 U/ml og penicillin and 100 µg/ml of streptomycin in a plastic Petri dish. The following day transfection was performed with 20 µg of DNA. The next day the cells were seeded in Petri dishes each containing 2 % of the cells. After 24 hours selection of transfectants was started using the above mentioned medium with the addition of 1 µM methotrexate. Selected clones were transferred to tissue culture flasks using cloning rings and tested for VCAM expression using the procedure described in Example 3. Ten clones tested all showed VCAM-1 expression.

Granulocytes were prepared by dextran sedimentation followed by percoll gradient centrifugation. Briefly, 40 ml of heparinized blood was mixed with an equal amount of dextrane solution (2 g/100 ml Dextrane T-500 (Pharmacia), 0.9 g/100 ml NaCl). After incubation for 30 min. at room temperature, the supernatant was centrifuged for 5 min. at 250 g. The pellet was resuspended in PBS to a cell concentration of 8.3 x 10^6/ml. A Percoll gradient was prepared containing 72, 68, 66, 63, 59 and 54 % Percol (100 % = Percoll 1.131 g/ml) with a total volume of 8 ml. 3 ml of cell suspension was placed on top of the gradient and centrifuged for 35 minutes at 600 g and room

temperature. After centrifugation the cell bands were aspirated, washed, and resuspended in PBS. Differential counts of the various preparations were performed by May-Grünwald staining. Granulocyte fractions containing 16.0% eosinophils, 82.8% neutrophils and 1.2% mononuclear cells were used in the following experiments.

The day before adherence experiments 25,000 untransfected or VCAM-1 transfected BHK cells were seeded in 24 well cell culture plates (NUNC, Denmark) in DMEM medium with methotrexate as described above. The following day granulocytes were prepared from a normal volunteer as described above and resuspended to a cell concentration of 0.27×10^6 cells/ml in assay buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Transfected and untransfected BHK cells were washed twice with assay buffer and incubated with 1 ml of the granulocyte cell suspension at 37°C for 30 minutes. The cells were washed twice with assay buffer, scraped off, lysed with 0.5 % N-cetyl-N,N,N-trimethylammoniumbromide (CTAB) and centrifuged. The supernatants were assayed by radioimmunoassay for the eosinophil specific protein eosinophil cationic protein (ECP) as described (P. Venge et al., Br. J. Haematol. 27, 1977, pp. 331-335). Similarly the supernatants were assayed by RIA for the neutrophil specific protein myeloperoxidase (MPO) (T. Oloffson et al., Scand. J. Haematol. 18, 1977, pp. 73-88.

The results of this experiment are shown in Table I. When transfected and non-transfected cells were lysed and assayed as described above, no ECP or MPO could be detected (results not shown).

As appears from Table I eosinophils adhere strongly to VCAM-1 expressing BHK cells, in contrast to neutrophils, which show no adhesion above background levels. Similar experiments were performed with granulocytes from 3 asthma patients with high eosinophil counts with essentially similar results.

Cell fraction	added x 10^{-6}	added x 10^{-6}	Eosinophils ECP ng	Eosinophils ECP %	Neutrophils MPO ng	Neutrophils MPO %
Granulocyte start fraction	0.043	0.222	100	435	1033	100
Granulocytes adhering to VCAM-1 cells	0.043	0.222	173	39.7	89	8.6
Granulocytes adhering to control cells	0.043	0.222	45.3	10.4	57	5.5

CLAIMS

1. A pharmaceutical composition for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface vascular cell adhesion molecules, the composition comprising a vascular cell adhesion molecule (VCAM) and a pharmaceutically acceptable carrier or excipient.

2. A composition according to claim 1, wherein the VCAM is in soluble form.

3. A composition according to claim 1 or 2, wherein the VCAM is a VCAM variant comprising seven immunoglobulin (Ig)-like domains, or a derivative thereof.

4. A composition according to claim 3, wherein the VCAM variant comprises the amino acid sequence shown in Figs. 1 A-E or 3 A-D appended hereto, or a derivative thereof.

5. A composition according to claim 3, wherein one or more of the Ig-like domains 1, 2, 3, 5, 6 and 7 of the VCAM variant are deleted.

6. A composition according to any of claims 1-5, wherein the VCAM is fused to another Ig superfamily molecule or a fragment thereof.

7. A composition according to any of claims 1-6 for the prophylaxis or treatment of allergic diseases or conditions, such as asthma, inflammatory bowel disease or dermatitis.

8. A composition according to any of claims 1-6 for the prophylaxis or treatment of autoimmune diseases, such as rheumatoid arthritis or diabetes.

9. A pharmaceutical composition for the prophylaxis or

treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface vascular cell adhesion molecules, the composition comprising an antibody which is capable of binding a VCAM and a pharmaceutically acceptable excipient or carrier.

10. A composition according to claim 9, wherein the antibody is one which is capable of specifically binding a VCAM variant comprising seven Ig-like domains.

11. A composition according to claim 10 comprising an antibody which reacts specifically with an epitope at least partially included in the following amino acid sequence
Glu Lys Pro Phe Thr Val Glu Ile Ser Pro Gly Pro Arg Ile Ala Ala Gln Ile Gly Asp Ser Val Met Leu Thr Cys Ser Val Met Gly Cys Glu Ser Pro Ser Phe Ser Trp Arg Thr Gln Ile Asp Ser Pro Leu Asn Gly Lys Val Arg Ser Glu Gly Thr Asn Ser Thr Leu Thr Leu Ser Pro Val Ser Phe Glu Asn Glu His Ser Tyr Leu Cys Thr Val Thr Cys Gly His Lys Lys Leu Glu Lys Gly Ile Gln Val Glu Leu Tyr Ser

12. A composition according to any of claims 9-11, wherein the antibody is a monoclonal antibody or a fragment thereof.

13. A method of screening for antagonists of VCAM binding to eosinophils, the method comprising incubating a VCAM or a cell capable of expressing a VCAM with a substance suspected to be an antagonist of VCAM binding and subsequently with eosinophils or a ligand derived from eosinophils and capable of binding VCAMs, and detecting any binding of the eosinophils or ligand to the VCAM, decreased binding indicating that said substance is an antagonist of VCAM binding.

14. A method of screening for antagonists of VCAM binding to eosinophils, the method comprising incubating eosinophils or a ligand derived from eosinophils and capable of binding VCAMs with a substance suspected to be an antagonist of VCAM binding and subsequently with a VCAM or a cell capable of expressing

VCAMs, and detecting any binding of the eosinophils or ligand to the VCAM, decreased binding indicating that said substance is an antagonist of VCAM binding.

15. A method according to claim 13 or 14, wherein the ligand derived from eosinophils capable of binding the VCAM variant is very late antigen-4 (VLA-4).

16. A method according to claim 13 or 14, wherein the VCAM is a VCAM variant comprising seven Ig-like domains, or a derivative thereof.

17. A method according to claim 16, wherein the VCAM variant comprises the amino acid sequence shown in the appended Figs. 1 A-E or 3 A-D, or a derivative thereof.

18. A method of preventing or treating a disease or condition involving the binding of eosinophils to cells expressing surface VCAMs, the method comprising administering, to a patient in need thereof, an effective dosage of a pharmaceutical composition according to any of claims 1-8 or 9-12.

19. A method according to claim 18, wherein the disease or condition is an allergic disease or condition, such as asthma, inflammatory bowel disease or dermatitis, or an autoimmune disease, such as rheumatoid arthritis or diabetes.

20. Use of a VCAM for the preparation of a medicament for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface VCAMs.

21. Use according to claim 20, wherein the VCAM is a VCAM variant comprising seven Ig-like domains, or a derivative thereof.

22. Use according to claim 20 or 21, wherein the VCAM is in

soluble form.

23. Use according to claim 21 or 22, wherein the VCAM variant comprises the amino acid sequence shown in the appended Figs. 1 A-E or 3 A-D, or a derivative thereof,.

24. Use according to any of claims 20-23, wherein the disease or condition is an allergic disease or condition, such as asthma, inflammatory bowel disease or dermatitis, or an autoimmune disease, such as rheumatoid arthritis or diabetes.

25. Use of an antibody capable of binding to a VCAM for the preparation of a medicament for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface VCAMs.

26. Use according to claim 25, wherein the antibody is capable of binding specifically to a VCAM variant comprising seven Ig-like domains, or a derivative thereof.

27. Use according to claim 25 or 26, wherein the disease or condition is an allergic disease or condition, such as asthma, inflammatory bowel disease or dermatitis, or an autoimmune disease, such as rheumatoid arthritis or diabetes.

28. Use of a VCAM antagonist isolated by the method of any of claims 13-17 for the preparation of a medicament for the prophylaxis or treatment of diseases or conditions involving the binding of eosinophils to cells expressing surface VCAMs.

29. Use of a VCAM derivative on which the ligand-binding site has been identified for establishing the three-dimensional structure of the ligand-binding site and designing a substance which is an antagonist of VCAM binding to cellular ligands, which substance has a three-dimensional structure which is substantially complementary to the three-dimensional structure of the ligand-binding site on the VCAM.

```
                                                                    ATGCCTGGGAAGATGGTCGTGATCCTTGGAGCCTCAAATATACTTTGGATAATGTTTGCA
    1 ----------+----------+----------+----------+----------+----------+ 60
      TACGGACCCTTCTACCAGCACTAGGAACCTCGGAGTTTATATGAAACCTATTACAAACGT

                                                                      MetProGlyLysMetValValIleLeuGlyAlaSerAsnIleLeuTrpIleMetPheAla -

                                                                    GCTTCTCAAGCTTTTAAAATCGAGACCACCCCAGAATCTAGATATCTTGCTCAGATTGGT
   61 ----------+----------+----------+----------+----------+----------+ 120
      CGAAGAGTTCGAAAATTTTAGCTCTGGTGGGGTCTTAGATCTATAGAACGAGTCTAACCA

                                                                      AlaSerGlnAlaPheLysIleGluThrThrProGluSerArgTyrLeuAlaGlnIleGly -

                                                                    GACTCCGTCTCATTGACTTGCAGCACCACAGGCTGTGAGTCCCCATTTTTCTCTTGGAGA
  121 ----------+----------+----------+----------+----------+----------+ 180
      CTGAGGCAGAGTAACTGAACGTCGTGGTGTCCGACACTCAGGGGTAAAAAGAGAACCTCT

                                                                      AspSerValSerLeuThrCysSerThrThrGlyCysGluSerProPhePheSerTrpArg -

                                                                    ACCCAGATAGATAGTCCACTGAATGGGAAGGTGACGAATGAGGGGACCACATCTACGCTG
  181 ----------+----------+----------+----------+----------+----------+ 240
      TGGGTCTATCTATCAGGTGACTTACCCTTCCACTGCTTACTCCCCTGGTGTAGATGCGAC

                                                                      ThrGlnIleAspSerProLeuAsnGlyLysValThrAsnGluGlyThrThrSerThrLeu -

                                                                    ACAATGAATCCTGTTAGTTTTGGGAACGAGCACTCTTACCTGTGCACAGCAACTTGTGAA
  241 ----------+----------+----------+----------+----------+----------+ 300
      TGTTACTTAGGACAATCAAAACCCTTGCTCGTGAGAATGGACACGTGTCGTTGAACACTT

                                                                      ThrMetAsnProValSerPheGlyAsnGluHisSerTyrLeuCysThrAlaThrCysGlu -

                                                                    TCTAGGAAATTGGAAAAAGGAATCCAGGTGGAGATCTACTCTTTTCCTAAGGATCCAGAG
  301 ----------+----------+----------+----------+----------+----------+ 360
      AGATCCTTTAACCTTTTTCCTTAGGTCCACCTCTAGATGAGAAAGGATTCCTAGGTCTC

                                                                      SerArgLysLeuGluLysGlyIleGlnValGluIleTyrSerPheProLysAspProGlu -
```

Fig. 1a

```
                ATTCATTTGAGTGGCCCTCTGGAGGCTGGGAAGCCGATCACAGTCAAGTGTTCAGTTGCT
361 ----------+----------+----------+----------+----------+----------+ 420
                TAAGTAAACTCACCGGGAGACCTCCGACCCTTCGGCTAGTGTCAGTTCACAAGTCAACGA

                IleHisLeuSerGlyProLeuGluAlaGlyLysProIleThrValLysCysSerValAla -

                GATGTATACCCATTTGACAGGCTGGAGATAGACTTACTGAAAGGAGATCATCTCATGAAG
421 ----------+----------+----------+----------+----------+----------+ 480
                CTACATATGGGTAAACTGTCCGACCTCTATCTGAATGACTTTCCTCTAGTAGAGTACTTC

                AspValTyrProPheAspArgLeuGluIleAspLeuLeuLysGlyAspHisLeuMetLys -

                AGTCAGGAATTTCTGGAGGATGCAGACAGGAAGTCCCTGGAAACCAAGAGTTTGGAAGTA
481 ----------+----------+----------+----------+----------+----------+ 540
                TCAGTCCTTAAAGACCTCCTACGTCTGTCCTTCAGGGACCTTTGGTTCTCAAACCTTCAT

                SerGlnGluPheLeuGluAspAlaAspArgLysSerLeuGluThrLysSerLeuGluVal -

                ACCTTTACTCCTGTCATTGAGGATATTGGAAAAGTTCTTGTTTGCCGAGCTAAATTACAC
541 ----------+----------+----------+----------+----------+----------+ 600
                TGGAAATGAGGACAGTAACTCCTATAACCTTTTCAAGAACAAACGGCTCGATTTAATGTG

                ThrPheThrProValIleGluAspIleGlyLysValLeuValCysArgAlaLysLeuHis -

                ATTGATGAAATGGATTCTGTGCCCACAGTAAGGCAGGCTGTAAAAGAATTGCAAGTCTAC
601 ----------+----------+----------+----------+----------+----------+ 660
                TAACTACTTTACCTAAGACACGGGTGTCATTCCGTCCGACATTTTCTTAACGTTCAGATG

                IleAspGluMetAspSerValProThrValArgGlnAlaValLysGluLeuGlnValTyr -

                ATATCACCCAAGAATACAGTTATTTCTGTGAATCCATCCACACAAAGCTGCAAGAAGGTGGC
661 ----------+----------+----------+----------+----------+----------+ 720
                TATAGTGGGTTCTTATGTCAATAAAGACACTTAGGTAGGTGTTTCGACGTTCTTCCACCG

                IleSerProLysAsnThrValIleSerValAsnProSerThrLysLeuGlnGluGlyGly -
```

Fig. 1b

```
     TCTGTGACCATGACCTGTTCCAGCGAGGGTCTACCAGCTCCAGAGATTTTCTGGAGTAAG
 721 ---------+---------+---------+---------+---------+---------+ 780
     AGACACTGGTACTGGACAAGGTCGCTCCCAGATGGTCGAGGTCTCTAAAAGACCTCATTC

     SerValThrMetThrCysSerSerGluGlyLeuProAlaProGluIlePheTrpSerLys -

     AAATTAGATAATGGGAATCTGCAGCACCTTTCTGGAAATGCAACTCTCACCTTAATTGCT
 781 ---------+---------+---------+---------+---------+---------+ 840
     TTTAATCTATTACCCTTAGACGTCGTGGAAAGACCTTTACGTTGAGAGTGGAATTAACGA

     LysLeuAspAsnGlyAsnLeuGlnHisLeuSerGlyAsnAlaThrLeuThrLeuIleAla -

     ATGAGGATGGAAGATTCTGGAATTTATGTGTGTGAAGGAGTTAATTTGATTGGGAAAAAC
 841 ---------+---------+---------+---------+---------+---------+ 900
     TACTCCTACCTTCTAAGACCTTAAATACACACACTTCCTCAATTAAACTAACCCTTTTTG

     MetArgMetGluAspSerGlyIleTyrValCysGluGlyValAsnLeuIleGlyLysAsn -

     AGAAAAGAGGTGGAATTAATTGTTCAAGAGAAACCATTTACTGTTGAGATCTCCCCTGGA
 901 ---------+---------+---------+---------+---------+---------+ 960
     TCTTTTCTCCACCTTAATTAACAAGTTCTCTTTGGTAAATGACAACTCTAGAGGGGACCT

     ArgLysGluValGluLeuIleValGlnGluLysProPheThrValGluIleSerProGly -

     CCCCGGATTGCTGCTCAGATTGGAGACTCAGTCATGTTGACATGTAGTGTCATGGGCTGT
 961 ---------+---------+---------+---------+---------+---------+ 1020
     GGGGCCTAACGACGAGTCTAACCTCTGAGTCAGTACAACTGTACATCACAGTACCCGACA

     ProArgIleAlaAlaGlnIleGlyAspSerValMetLeuThrCysSerValMetGlyCys -

     GAATCCCCATCTTTCTCCTGGAGAACCCAGATAGACAGCCCTCTGAACGGGAAGGTGAGG
1021 ---------+---------+---------+---------+---------+---------+ 1080
     CTTAGGGGTAGAAAGAGGACCTCTTGGGTCTATCTGTCGGGAGACTTGCCCTTCCACTCC

     GluSerProSerPheSerTrpArgThrGlnIleAspSerProLeuAsnGlyLysValArg -
```

Fig. 1c

```
                                                                  AGTGAGGGGACCAATTCCACGCTGACCCTGAGCCCTGTGAGTTTTGAGAACGAACACTCT
1081 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1140
                TCACTCCCCTGGTTAAGGTGCGACTGGGACTCGGGACACTCAAAACTCTTGCTTGTGAGA

                                                                     SerGluGlyThrAsnSerThrLeuThrLeuSerProValSerPheGluAsnGluHisSer -

                                                                  TATCTGTGCACAGTGACTTGTGGACATAAGAAACTGGAAAAGGGAATCCAGGTGGAGCTC
1141 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1200
                ATAGACACGTGTCACTGAACACCTGTATTCTTTGACCTTTTCCCTTAGGTCCACCTCGAG

                                                                     TyrLeuCysThrValThrCysGlyHisLysLysLeuGluLysGlyIleGlnValGlyGluLeu -

                                                                  TACTCATTCCCTAGAGATCCAGAAATCGAGATGAGTGGTGGCCTCGTGAATGGGAGCTCT
1201 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1260
                ATGAGTAAGGGATCTCTAGGTCTTTAGCTCTACTCACCACCGGAGCACTTACCCTCGAGA

                                                                     TyrSerPheProArgAspProGluIleGluMetSerGlyGlyLeuValAsnGlySerSer -

                                                                  GTCACTGTAAGCTGCAAGGTTCCTAGCGTGTACCCCCTTGACCGGCTGGAGATTGAATTA
1261 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1320
                CAGTGACATTCGACGTTCCAAGGATCGCACATGGGGGAACTGGCCGACCTCTAACTTAAT

                                                                     ValThrValSerCysLysValProSerValTyrProLeuAspArgLeuGluIleGluLeu -

                                                                  CTTAAGGGGGAGACTATTCTGGAGAATATAGAGTTTTTGGAGGATACGGATATGAAATCT
1321 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1380
                GAATTCCCCCTCTGATAAGACCTCTTATATCTCAAAAACCTCCTATGCCTATACTTTAGA

                                                                     LeuLysGlyGluThrIleLeuGluAsnIleGluPheLeuGluAspThrAspMetLysSer -

                                                                  CTAGAGAACAAAAGTTTGGAAATGACCTTCATCCCTACCATTGAAGATACTGGAAAAGCT
1381 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1440
                GATCTCTTGTTTTCAAACCTTTACTGGAAGTAGGGATGGTAACTTCTATGACCTTTTCGA

                                                                     LeuGluAsnLysSerLeuGluMetThrPheIleProThrIleGluAspThrGlyLysAla -
```

Fig. 1d

```
                                                                  CTTGTTTGTCAGGCTAAGTTACATATTGATGACATGGAATTCGAACCCAAACAAAGGCAG
1441 ----------+----------+----------+----------+----------+----------+ 1500
                GAACAAACAGTCCGATTCAATGTATAACTACTGTACCTTAAGCTTGGGTTTGTTTCCGTC

                LeuValCysGlnAlaLysLeuHisIleAspAspMetGluPheGluProLysGlnArgGln -

                AGTACGCAAACACTTTATGTCAATGTTGCCCCCAGAGATACAACCGTCTTGGTCAGCCCT
1501 ----------+----------+----------+----------+----------+----------+ 1560
                TCATGCGTTTGTGAAATACAGTTACAACGGGGGTCTCTATGTTGGCAGAACCAGTCGGGA

                SerThrGlnThrLeuTyrValAsnValAlaProArgAspThrThrValLeuValSerPro -

                TCCTCCATCCTGGAGGAAGGCAGTTCTGTGAATATGACATGCTTGAGCCAGGGCTTTCCT
1561 ----------+----------+----------+----------+----------+----------+ 1620
                AGGAGGTAGGACCTCCTTCCGTCAAGACACTTATACTGTACGAACTCGGTCCCGAAAGGA

                SerSerIleLeuGluGluGlySerSerValAsnMetThrCysLeuSerGlnGlyPhePro -

                GCTCCGAAAATCCTGTGGAGCAGGCAGCTCCCTAACGGGGAGCTACAGCCTCTTTCTGAG
1621 ----------+----------+----------+----------+----------+----------+ 1680
                CGAGGCTTTTAGGACACCTCGTCCGTCGAGGGATTGCCCCTCGATGTCGGAGAAAGACTC

                AlaProLysIleLeuTrpSerArgGlnLeuProAsnGlyGluLeuGlnProLeuSerGlu -

                AATGCAACTCTCACCTTAATTTCTACAAAAATGGAAGATTCTGGGGTTTATTTATGTGAA
1681 ----------+----------+----------+----------+----------+----------+ 1740
                TTACGTTGAGAGTGGAATTAAAGATGTTTTTACCTTCTAAGACCCCAAATAAATACACTT

                AsnAlaThrLeuIleSerThrLysMetGluAspSerGlyValTyrLeuCysGlu -

                GGAATTAACCAGGCTGGAAGAAGGCAGAAAGGAAGTGGAATTAATTATCCGAGTTACTCCA
1741 ----------+----------+----------+----------+----------+----------+ 1800
                CCTTAATTGGTCCGACCTTCTTCGTCTTTCCTTCACCTTAATTAATAGGCTCAATGAGGT

                GlyIleAsnGlnAlaGlyArgSerArgLysGluValGluLeuIleIleArgValThrPro -
```

Fig. 1e

```
                                                                  AAAGACATAAAACTTACAGCTTTTCCTTCTGAGAGTGTCAAAGAAGGAGACACTGTCATC
1801 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1860
                TTTCTGTATTTTGAATGTCGAAAAGGAAGACTCTCACAGTTTCTTCCTCTGTGACAGTAG

                                                                     LysAspIleLysLeuThrAlaPheProSerGluSerValLysGluGlyAspThrValIle -

                                                                  ATCTCTTGTACATGTGGAAATGTTCCAGAAACATGGATAATCCTGAAGAAAAAAGCGGAG
1861 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1920
                TAGAGAACATGTACACCTTTACAAGGTCTTTGTACCTATTAGGACTTCTTTTTTCGCCTC

                                                                     IleSerCysThrCysGlyAsnValProGluThrTrpIleIleLeuLysLysAlaGlu -

                                                                  ACAGGAGACACAGTACTAAAATCTATAGATGGCGCCTATACCATCCGAAAGGCCCAGTTG
1921 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1980
                TGTCCTCTGTGTCATGATTTTAGATATCTACCGCGGATATGGTAGGCTTTCCGGGTCAAC

                                                                     ThrGlyAspThrValLeuLysSerIleAspGlyAlaTyrThrIleArgLysAlaGlnLeu -

                                                                  AAGGATGCGGGAGTATATGAATGTGAATCTAAAAACAAAGTTGGCTCACAATTAAGAAGT
1981 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 2040
                TTCCTACGCCCTCATATACTTACACTTAGATTTTTGTTTCAACCGAGTGTTAATTCTTCA

                                                                     LysAspAlaGlyValTyrGluCysGluSerLysAsnLysValGlySerGlnLeuArgSer -

                                                                  TTAACACTTGATGTTCAAGGAAGAGAAAACAACAAAGACTATTTTTCTCCTGAGCTTCTC
2041 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 2100
                AATTGTGAACTACAAGTTCCTTCTCTTTTGTTGTTTCTGATAAAAGAGGACTCGAAGAG

                                                                     LeuThrLeuAspValGlnGlyArgGluAsnAsnLysAspTyrPheSerProGluLeuLeu -

                                                                  GTGCTCTATTTTGCATCCTCCTTAATAATACCTGCCATTGGAATGATAATTACTTTGCA
2101 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 2160
                CACGAGATAAAACGTAGGAGGAATTATTATGGACGGTAACCTTACTATTAAATGAAACGT

                                                                     ValLeuTyrPheAlaSerSerLeuIleIleProAlaIleGlyMetIleIleTyrPheAla -
```

Fig. 1f

```
                AGAAAAGCCAACATGAAGGGGTCATATAGTCTTGTAGAAGCACAGAAATCAAAAGTGTAG
2161 ----------+----------+----------+----------+----------+----------+ 2220
                TCTTTTCGGTTGTACTTCCCCAGTATATCAGAACATCTTCGTGTCTTTAGTTTTCACATC

                ArgLysAlaAsnMetLysGlySerTyrSerLeuValGluAlaGlnLysSerLysValEnd -
```

Fig. 1g

```
                .         .         .         .         .
  1 ....................EKPFTVEISPGPRIAAQIGDSVMLTCSVM 29
                                |   |   |   |  |||||||  ||||
  1 MPGKMVVILGASNILWIMFAASQAFKIETTPESRYLAQIGDSVSLTCSTT 50
                .         .         .         .         .
 30 GCESPSFSWRTQIDSPLNGKVRSEGTNSTLTLSPVSFENEHSYLCTVTCG 79
     |||||  ||||||||||||||||  |||  ||||  ||||  ||||||||  ||
 51 GCESPFFSWRTQIDSPLNGKVTNEGTTSTLTMNPVSFGNEHSYLCTATCE 100
                .         .         .         .         .
 80 HKKLEKGIQVELYS..................................... 93
       |||||||||  ||
101 SRKLEKGIQVEIYSFPKDPEIHLSGPLEAGKPITVKCSVADVYPFDRLEI 150
```

Fig. 2

```
                                                                    ATGCCTGGGAAGATGGTCGTGATCCTTGGAGCCTCAAATATACTTTGGATAATGTTTGCA
    1 ----------+----------+----------+----------+----------+----------+ 60
                                                                    TACGGACCCTTCTACCAGCACTAGGAACCTCGGAGTTTATATGAAACCTATTACAAACGT

                                                                    MetProGlyLysMetValValIleLeuGlyAlaSerAsnIleLeuTrpIleMetPheAla -

                                                                    GCTTCTCAAGCTTTTAAAATCGAGACCACCCCAGAATCTAGATATCTTGCTCAGATTGGT
   61 ----------+----------+----------+----------+----------+----------+ 120
                                                                    CGAAGAGTTCGAAAATTTAGCTCTGGTGGGGTCTTAGATCTATAGAACGAGTCTAACCA

                                                                    AlaSerGlnAlaPheLysIleGluThrThrProGluSerArgTyrLeuAlaGlnIleGly -

                                                                    GACTCCGTCTCATTGACTTGCAGCACCACAGGCTGTGAGTCCCCATTTTTCTCTTGGAGA
  121 ----------+----------+----------+----------+----------+----------+ 180
                                                                    CTGAGGCAGAGTAACTGAACGTCGTGGTGTCCGACACTCAGGGGTAAAAAGAGAACCTCT

                                                                    AspSerValSerLeuThrCysSerThrThrGlyCysGluSerProPhePheSerTrpArg -

                                                                    ACCCAGATAGATAGTCCACTGAATGGGAAGGTGACGAATGAGGGGACCACATCTACGCTG
  181 ----------+----------+----------+----------+----------+----------+ 240
                                                                    TGGGTCTATCTATCAGGTGACTTACCCTTCCACTGCTTACTCCCCTGGTGTAGATGCGAC

                                                                    ThrGlnIleAspSerProLeuAsnGlyLysValThrAsnGluGluTyrThrSerThrLeu -

                                                                    ACAATGAATCCTGTTAGTTTTGGGAACGAGCACTCTTACCTGTGCACAGCAACTTGTGAA
  241 ----------+----------+----------+----------+----------+----------+ 300
                                                                    TGTTACTTAGGACAATCAAAACCCTTGCTCGTGAGAATGGACACGTGTCGTTGAACACTT

                                                                    ThrMetAsnProValSerPheGlyAsnGluHisSerTyrLeuCysThrAlaThrCysGlu -

                                                                    TCTAGGAAATTGGAAAAAGGAATCCAGGTGGAGATCTACTCTTTCCTAAGGATCCAGAG
  301 ----------+----------+----------+----------+----------+----------+ 360
                                                                    AGATCCTTTAACCTTTTCCTTAGGTCCACCTCTAGATGAGAAAAGGATTCCTAGGTCTC

                                                                    SerArgLysLeuGluLysGlyIleGlnValGluIleTyrSerPheProLysAspProGlu -
```

Fig. 3a

```
                ATTCATTTGAGTGGCCCTCTGGAGGCTGGGAAGCCGATCACAGTCAAGTGTTCAGTTGCT
361 ------------+----------+----------+----------+----------+----------+ 420
                TAAGTAAACTCACCGGGAGACCTCCGACCCTTCGGCTAGTGTCAGTTCACAAGTCAACGA

                  IleHisLeuSerGlyProLeuGluAlaGlyLysProIleThrValLysCysSerValAla -

                GATGTATACCCATTTGACAGGCTGGAGATAGACTTACTGAAGGAGATCATCTCATGAAG
421 ------------+----------+----------+----------+----------+----------+ 480
                CTACATATGGGTAAACTGTCCGACCTCTATCTGAATGACTTTCCTCTAGTAGAGTACTTC

                  AspValTyrProPheAspArgLeuGluIleAspLeuLysGlyAspHisLeuMetLys -

                AGTCAGGAATTTCTGGAGGATGCAGACAGGAAGTCCCTGGAAACCAAGAGTTTGGAAGTA
481 ------------+----------+----------+----------+----------+----------+ 540
                TCAGTCCTTAAAGACCTCCTACGTCTGTCCTTCAGGGACCTTTGGTTCTCAAACCTTCAT

                  SerGlnGluPheLeuGluAspAlaAspArgLysSerLeuGluThrLysSerLeuGluVal -

                ACCTTTACTCCTGTCATTGAGGATATTGGAAAAGTTCTTGTTTGCCGAGCTAAATTACAC
541 ------------+----------+----------+----------+----------+----------+ 600
                TGGAAATGAGGACAGTAACTCCTATAACCTTTTCAAGAACAAACGGCTCGATTTAATGTG

                  ThrPheThrProValIleGluAspIleGlyLysValLeuValCysArgAlaLysLeuHis -

                ATTGATGAAATGGATTCTGTGCCCACAGTAAGGCAGGCTGTAAAAGAATTGCAAGTCTAC
601 ------------+----------+----------+----------+----------+----------+ 660
                TAACTACTTTACCTAAGACACGGGTGTCATTCCGTCCGACATTTCTTAACGTTCAGATG

                  IleAspGluMetAspSerValProThrValArgGlnAlaValLysGluLeuGlnValTyr -

                ATATCACCCAAGAAATACAGTTATTTCTGTGAATCCATCCACAAAGCTGCAAGAAGGTGGC
661 ------------+----------+----------+----------+----------+----------+ 720
                TATAGTGGGTTCTTATGTCAATAAAGACACTTAGGTAGGTGTTTCGACGTTCTTCCACCG

                  IleSerProLysAsnThrValIleSerValAsnProSerThrLysLeuGlnGluGlyGly -
```

Fig. 3b

```
                                                                    TCTGTGACCATGACCTGTTCCAGCGAGGGTCTACCAGCTCCAGAGATTTTCTGGAGTAAG
721 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 780
              AGACACTGGTACTGGACAAGGTCGCTCCCAGATGGTCGAGGTCTCTAAAAGACCTCATTC

                                                                    SerValThrMetThrCysSerSerGluGlyLeuProAlaProGluIlePheTrpSerLys -

                                                                    AAATTAGATAATGGGAATCTGCAGCACCTTTCTGGAAATGCAACTCTCACCTTAATTGCT
781 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 840
              TTTAATCTATTACCCTTAGACGTCGTGGAAAGACCTTTACGTTGAGAGTGGGAATTAACGA

                                                                    LysLeuAspAsnGlyAsnLeuGlnHisLeuSerGlyAsnAlaThrLeuThrLeuIleAla -

                                                                    ATGAGGATGGAAGATTCTGGAATTTATGTGTGTGAAGGAGTTAATTTGATTGGGAAAAAC
841 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 900
              TACTCCTACCTTCTAAGACCTTAAATACACACACACTTCCTCAATTAAACTAACCCTTTTTG

                                                                    MetArgMetGluAspSerGlyIleTyrValCysGluGlyValAsnLeuIleGlyLysAsn -

                                                                    AGAAAAGAGGTGGAATTAATTGTTCAAGAGAAACCATTTACTGTTGAGATCTCCCCTGGA
901 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 960
              TCTTTTCTCCACCTTAATTAACAAGTTCTCTTTGGTAAATGACAACTCTAGAGGGGACCT

                                                                    ArgLysGluValGluLeuIleValGlnGluLysProPheThrValGluIleSerProGly -

                                                                    CCCCGGATTGCTGCTCAGATTGGAGACTCAGTCATGTTGACATGTAGTGTCATGGGCTGT
961 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1020
              GGGGCCTAACGACGAGTCTAACCTCTGAGTCAGTACAACTGTACATCACAGTACCCGACA

                                                                    ProArgIleAlaAlaGlnIleGlyAspSerValMetLeuThrCysSerValMetGlyCys -

                                                                    GAATCCCCATCTTTCTCCTGGAGAACCCAGATAGACAGCCCTCTGAACGGGAAGGTGAGG
1021 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1080
               CTTAGGGGTAGAAAGAGGACCTCTTGGGTCTATCTGTCGGGAGACTTGCCCTTCCACTCC

                                                                    GluSerProSerPheSerTrpArgThrGlnIleAspSerProLeuAsnGlyLysValArg -
```

Fig. 3c

```
                                                                  AGTGAGGGGACCAATTCCACGCTGACCCTGAGCCCTGTGAGTTTTGAGAACGAACACTCT
1081 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1140
                TCACTCCCCTGGTTAAGGTGCGACTGGGACTCGGGACACTCAAAACTCTTGCTTGTGAGA

                SerGluGlyThrAsnSerThrLeuThrLeuSerProValSerPheGluAsnGluHisSer

                TATCTGTGCACAGTGACTTGTGGACATAAGAAACTGGAAAAGGGAATCCAGGTGGAGCTC
1141 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1200
                ATAGACACGTGTCACTGAACACCTGTATTCTTTGACCTTTTCCCTTAGGTCCACCTCGAG

                TyrLeuCysThrValThrCysGlyHisLysLysLeuGluLysGlyIleGlnValGlnLeu -

                TACTCATTCCCTAGAGATCCAGAAATCGAGATGAGTGGTGGCCTCGTGAATGGGAGCTCT
1201 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1260
                ATGAGTAAGGGATCTCTAGGTCTTTAGCTCTACTCACCACCGGAGCACTTACCCTCGAGA

                TyrSerPheProArgAspProGluIleGluMetSerGlyGlyLeuValAsnGlySerSer -

                GTCACTGTAAGCTGCAAGGTTCCTAGCGTGTACCCCCTTGACCGGCTGGAGATTGAATTA
1261 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1320
                CAGTGACATTCGACGTTCCAAGGATCGCACATGGGGGAACTGGCCGACCTCTAACTTAAT

                ValThrValSerCysLysValProSerValTyrProLeuAspArgLeuGluIleGluLeu -

                CTTAAGGGGGAGACTATTCTGGAGAATATAGAGTTTTTGGAGGATACGGATATGAAATCT
1321 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1380
                GAATTCCCCCTCTGATAAGACCTCTTATATCTCAAAAACCTCCTATGCCTATACTTTAGA

                LeuLysGlyGluThrIleLeuGluAsnIleGluPheLeuGluAspThrAspMetLysSer -

                CTAGAGAACAAAAGTTTGGAAATGACCTTCATCCCTACCATTGAAGATACTGGAAAAGCT
1381 ----------+----------+----------+----------+----------+----------+----------+----------+----------+----------+ 1440
                GATCTCTTGTTTTCAAACCTTTACTGGAAGTAGGGATGGTAACTTCTATGACCTTTTCGA

                LeuGluAsnLysSerLeuGluMetThrPheIleProThrIleGluAspThrGlyLysAla -
```

Fig. 3d

```
                CTTGTTTGTCAGGCTAAGTTACATATTGATGACATGGAATTCGAACCCAAACAAAGGCAG
1441  ----------+----------+----------+----------+----------+----------+ 1500
                GAACAAACAGTCCGATTCAATGTATAACTACTGTACCTTAAGCTTGGGTTTGTTTCCGTC

                LeuValCysGlnAlaLysLeuHisIleAspAspMetGluPheGluProLysGlnArgGln -

                AGTACGCAAACACTTTATGTCAATGTTGCCCCCAGAGATACAACCGTCTTGGTCAGCCCT
1501  ----------+----------+----------+----------+----------+----------+ 1560
                TCATGCGTTTGTGAAATACAGTTACAACGGGGGTCTCTATGTTGGCAGAACCAGTCGGGA

                SerThrGlnThrLeuTyrValAsnValAlaProArgAspThrThrValLeuValSerPro -

                TCCTCCATCCTGGAGGAAGGCAGTTCTGTGAATATGACATGCTTGAGCCAGGGCTTTCCT
1561  ----------+----------+----------+----------+----------+----------+ 1620
                AGGAGGTAGGACCTCCTTCCGTCAAGACACTTATACTGTACGAACTCGGTCCCGAAAGGA

                SerSerIleLeuGluGluGlySerSerValAsnMetThrCysLeuSerGlnGlyPhePro -

                GCTCCGAAAATCCTGTGGAGCAGGCAGCTCCCTAACGGGGAGGCTACAGCCTCTTTCTGAG
1621  ----------+----------+----------+----------+----------+----------+ 1680
                CGAGGCTTTTAGGACACCTCGTCCGTCGAGGGATTGCCCCTCGATGTCGGAGAAAGACTC

                AlaProLysIleLeuTrpSerArgGlnLeuProAsnGlyGluLeuGlnProLeuSerGlu -

                AATGCAACTCTCACCTTAATTTCTACAAAAATGGAAGATTCTGGGGTTTATTTATGTGAA
1681  ----------+----------+----------+----------+----------+----------+ 1740
                TTACGTTGAGAGTGGAATTAAAGATGTTTTTACCTTCTAAGACCCCAAATAAATACACTT

                AsnAlaThrLeuThrLeuIleSerThrLysMetGluAspSerGlyValTyrLeuCysGlu -

                GGAATTAACCAGGCTGGAAGAAGGAAGTGGAATTAATTATCCGAGTTACTCCA
1741  ----------+----------+----------+----------+----------+----------+ 1800
                CCTTAATTGGTCCGACCTTCTTCGTCTTTCCTTCACCTTAATTAATAGGCTCAATGAGGT

                GlyIleAsnGlnAlaGlyArgSerArgLysGluValGluLeuIleIleArgValThrPro -
```

Fig. 3e

```
1801  AAAGACATAAAACTTACAGCTTTTCCTTCTGAGAGTGTCAAAGAAGGAGACACTGTCATC
      ----------+----------+----------+----------+----------+----------+ 1860
      TTTCTGTATTTTGAATGTCGAAAAGGAAGACTCTCACAGTTTCTTCCTCTGTGACAGTAG

      LysAspIleLysLeuThrAlaPheProSerGluSerValLysGluGlyAspThrValIle -

1861  ATCTCTTGTACATGTGGAAATGTTCCAGAAACATGGATAATCCTGAAGAAAAAAGCGGAG
      ----------+----------+----------+----------+----------+----------+ 1920
      TAGAGAACATGTACACCTTTACAAGGTCTTTGTACCTATTAGGACTTCTTTTTTCGCCTC

      IleSerCysThrCysGlyAsnValProGluThrTrpIleIleLeuLysLysLysAlaGlu-

1921  ACAGGAGACACAGTACTAAAATCTATAGATGGCGCCTATACCATCCGAAAGGCCCAGTTG
      ----------+----------+----------+----------+----------+----------+ 1980
      TGTCCTCTGTGTCATGATTTTAGATATCTACCGCGGATATGGTAGGCTTTCCGGGTCAAC

      ThrGlyAspThrValLeuLysSerIleAspGlyAlaTyrThrIleArgLysAlaGlnLeu -

1981  AAGGATGCGGGAGTATATGAATGTGAATCTAAAAACAAAGTTGGCTCACAATTAAGAA
      ----------+----------+----------+----------+----------+----------+ 2040
      TTCCTACGCCCTCATATACTTACACTTAGATTTTTGTTTCAACCGAGTGTTAATTCTTCA

      LysAspAlaGlyValTyrGluCysGluSerLysAsnLysValGlySerGlnLeuArgSer -

2041  TTAACACTTGATGTTCAAGGAAGAGAAAACAACAAAGACTATTTTTCTTAG
      ----------+----------+----------+----------+----------+-- 2091
      AATTGTGAACTACAAGTTCCTTCTCTTTTGTTGTTTCTGATAAAAGAATC

      LeuThrLeuAspValGlnGlyArgGluAsnAsnLysAspTyrPheSerEnd -
```

Fig. 3f

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00193

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; G 01 N; C 12 Q; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP, A2, 0314863 (BAYLOR COLLEGE OF MEDICINE ET AL.) 10 May 1989, see esp. the claims --	1-2,7-9, 12-14, 18-19, 20-22, 25,27-28
Y	WO, A1, 9003400 (DANA-FARBER CANCER INSTITUTE) 5 April 1990, see esp. the claims --	1-2,7-9, 12-14, 18-19, 20-22, 25,27-28
Y	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07243285, Elices M.J. et al: "VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site", & Cell Feb 23 1990, 60 (4) p577-84 --	1-2,7-9, 12-14, 18-19, 20-22, 25,27-28

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17th December 1991	1991 -12- 18
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carolina Palmcrantz Carolina Palmcrantz

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0333517 (THE ROCKEFELLER UNIVERSITY) 20 September 1989, see esp. the abstract --	1-2,7-8
P,X	WO, A1, 9013300 (BIOGEN, INC.) 15 November 1990, see the whole document --	1-2,9, 12,20-22,25
T	J. Clin. Invest., Vol. 88, July 1991 A: Dobrina et al.: "Mechanisms of Eosinophil Adherence to Cultured Vascular Endothelial Cells", see page 20 - page 26 --	1-2

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 18-19 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [X] Claim numbers 2, 4-6, 21 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

and other claims as far as they include the too broadly formulated wordings "variant", "a derivative thereof", "is fused to another Ig superfamily molecule", "a fragment thereof".

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See the attached sheet.

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED

The subjects, as listed below, are so different from each other that no technical relationship can be appreciated to be present so as to form a single general inventive concept.

Invention A (claims 1-8,20-24) describes a pharmaceutical composition comprising a vascular cell adhesion molecule (VCAM).

Further, invention B (claims 9-12, 25-27 and partly 29) describes a pharmaceutical composition comprising an antibody which is capable of binding a VCAM.

Invention C (claims 13-17, 28 and partly 29) describes a method of screening for antagonists of VCAM binding to eosinophils.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 91/00193

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 31/10/91 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0314863	89-05-10	AU-D-	1550988	89-07-27
		AU-D-	2633388	89-07-27
		JP-A-	1135724	89-05-29
WO-A1- 9003400	90-04-05	AU-D-	4412889	90-04-18
		EP-A-	0365837	90-05-02
		JP-T-	3501861	91-04-25
		AU-D-	5129990	90-09-20
		JP-A-	3157397	91-07-05
EP-A2- 0333517	89-09-20	AU-D-	3149289	89-09-21
		JP-A-	2076898	90-03-16
WO-A1- 9013300	90-11-15	AU-D-	6049290	90-11-29